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Determining the Protective Effects of Quercetin Against Cadmium Toxicity in Human Embryonic Kidney Cells

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ABSTRACT

Cadmium is a toxic industrial and environmental pollutant found in groundwater, air, soils, food and cigarettes. Chronic intake of low levels of cadmium has been shown to result in renal dysfunction due to cell death which can occur via apoptosis as well as necrosis. Previous studies have shown that plant extracts containing quercetin, a flavonoid found in many fruits and vegetables, protect against cadmium toxicity in rat liver hepatocytes. To determine if quercetin may have a protective effect in a cadmium-treated human embryonic kidney cell line, HEK-293 cells were treated using concentrations of cadmium chloride from 10 to 50 μM for 24 hours. Using a cell proliferation assay, it was determined that cadmium chloride inhibited cellular proliferation in a concentration dependent manner. For further studies, 30 μM cadmium chloride was used since it inhibited growth by 31.98%. Pretreating cells for 24 hours before cadmium exposure with concentrations of 10 μM to 200 μM quercetin suggested that this flavonoid partially protects HEK-293 cells from cadmium toxicity at all levels of treatment. While these results suggest a protective effect, it is unclear if quercetin is inhibiting a cadmium-induced apoptotic or necrotic pathway. Previous studies have suggested that cadmium-induced apoptosis increases levels of p47phox, one of the subunits of NADPH oxidase. To determine if quercetin reduces p47phox expression, HEK-293 cells were pretreated for 24 hours before cadmium treatment, and immunoblot analysis was used to determine p47phox expression. Preliminary results suggest that quercetin does not reduce p47phox levels and therefore, may not specifically inhibit cadmium-induced apoptosis.

INTRODUCTION

In 2016, chronic kidney disease and/or failure was shown to affect 14% of adults in the United States (National Institute of Diabetes and Digestive and Kidney Diseases, 2016). More than 661,000 Americans are living with kidney failure today; of these, 468,000 individuals are on dialysis, and roughly 193,000 live with a functioning kidney transplant. Each year, kidney disease kills more people than breast or prostate cancer (Xu et al., 2013). For example, in 2013, more than 47,000 Americans died from kidney disease (Xu et al., 2013). Chronic kidney disease is not only a growing problem in United States, but is also a problem worldwide, specifically in areas with increased industrialization. Global chronic kidney disease is observed at a rate of approximately 11% (Hill et al., 2016). While chronic kidney disease is divided into five different stages, depending on severity, the disease usually goes undiagnosed showing no symptoms before reaching stage 4, which is identified by a glomerular filtration rate low enough to cause patients to begin dialysis (Hill et al., 2016).

The causes of chronic kidney disease are many including obesity, cardiovascular disease, as well as metal toxicity, one of which is cadmium. According to the International Cadmium Association, exposure to cadmium can have negative effects on a number of organs, including the kidney (2010). Inhalation can cause side effects almost immediately inducing inflammation and altering the mechanics of the respiratory system (Johri et al., 2010). Cadmium has also been shown to be involved in bone disease (International Cadmium Association, 2010). Long term exposure to cadmium has been shown to primarily affect the kidneys and the liver. Studies have revealed that once cadmium enters the body, it has the ability to remain in the kidneys for up to 30 years.

The presence of cadmium is rapidly increasing in the environment as rates of industrialization increase exponentially across the world. A recent study by the United States Geological Survey (USGS) revealed in its Mineral Commodity Report that China, the Republic of Korea, and Japan were the leading producers of cadmium in 2015. In these countries that produce high emissions, 600-2000 μg of cadmium can be released into the environment every day (Toxic Substances Portal, 2015). Even in areas with lower emission levels, 100-390 μg of cadmium can be released daily (Toxic Substances Portal, 2015). One route of emissions is through the production of nickel-cadmium (NiCd) based batteries, which accounted for more than 80% of cadmium global emissions in 2015. While these batteries require minimal costs for companies producing them, cadmium byproducts and emissions are greatly increasing throughout the world. This trend will likely continue since alternatives to lithium batteries are more expensive and would immensely increase production cost compared to NiCd products. Therefore, the risk of cadmium exposure will likely remain a threat to human health. Another environmental source of cadmium is through the use of cigarettes. A single cigarette contains 2 μg of cadmium alone, and when inhaled, 10-50% of cadmium in that cigarette goes directly to the lungs (Toxic Substances Portal, 2015). This can affect smokers as well as those exposed to second-hand smoke.

There are several routes in which cadmium can enter the body. The first of these is inhalation, which can occur through cadmium contaminated oxygen, as well as through cigarette smoke. Also, there is the possibility that factories within third world countries producing food products could also be emitting byproducts through production efforts, causing cross contamination of food and cadmium. Finally, when cadmium is emitted as a byproduct, it is often released into the air and can contaminate water sources and soil. Uptake of cadmium in

agricultural crops leads to cadmium being ingested by humans. No matter which method of exposure occurs, chronic intake of cadmium in food or from contaminated air supplies may result in organ malfunction (Fujiwara et al., 2012).

The mechanism by which cadmium enters the cell and induces organ failure has been heavily studied throughout recent years. Cadmium has been shown to enter the kidney, as well as other organs, through a variety of transport-binding proteins that have been characterized to play a role in renal cadmium accumulation (Yang and Shu, 2015). These include metallothionein, zinc transporters, calcium transporters, and divalent metal-ion transporter-1 (Yang and Shu, 2015). With chronic intoxication of cadmium, de novo synthesis of metallothionein is stimulated, suggesting that this transporter is primarily involved in cadmium removal (Sabolić et al., 2010). According to the Agency for Toxic Substances and Disease Registry, cadmium first accumulates in the blood by binding to metallothionein (MT), forming CdMT complexes that then travel to the kidney for degradation (Toxic Substances Portal, 2015). Once CdMT complexes reach the kidney, they are filtered and endocytosed by the proximal convoluted tubule and binding is broken down by lysosomes, releasing cadmium from metallothionein (Sabolić et al., 2010). Once cadmium is released, it can immediately affect the cells of the tubule (Sabolić et al., 2010). Mechanisms for CdMT binding as well as reasons for endocytosis in the proximal tubule are unknown thus far.

Once cadmium reaches the kidneys, it may alter cellular function through multiple mechanisms including oxidative stress. Oxidative stress refers to an increase in reactive oxygen species (ROS) within a cell. Low production of reactive oxygen species is actually necessary to cellular survival and maintaining homeostasis. ROS are normal byproducts of oxygen metabolism and have been shown to have important roles in the regulation of cellular defense,

hormone synthesis and signaling, activation of G protein-coupled receptors, and ion channels and kinases/phosphatases (Bhattacharyya et al., 2014). ROS also have an important role in the regulation of transcription factors and gene expression. However, high quantities of ROS may produce oxidative stress causing havoc throughout the cell as well as the rest of the body in terms of pathogenesis (Bhattacharyya et al., 2014).

While it is not clear how oxidative stress induced by cadmium toxicity results in cell death, mitochondria are thought to be a likely target of the reactive oxygen species cascade (Gobe and Crane, 2010). It has been shown that cadmium has the ability to penetrate the mitochondria (Lee, 2004), ultimately resulting in increased ROS production through a multitude of intermediate steps. This likely causes mitochondrial damage (Lee, 2004; Cannino et al., 2009) which can lead to apoptosis through multiple cellular processes: the loss of mitochondrial membrane potentially causes the release of cytochrome-c and activation of caspase pathways leading to apoptosis and/or efforts to remove malfunctioning mitochondria through autophagy could also lead to apoptosis (Gobe and Crane 2010). While cadmium is known to induce oxidative stress, the molecular mechanisms involved in the cell damage from oxidative stress in cadmium-induced chronic kidney disease are not well understood (Gobe and Crane, 2010). Cadmium-induced cellular apoptosis has been examined in a number of pathways targeting specific proteins. One set of targets include the subunits of NADPH oxidase, a protein that is involved in producing superoxides and serve as precursors to ROS. Cadmium-induced toxicity has been shown to increase NADPH oxidase levels, providing a possible mechanism in which ROS levels could contribute to cell death by cadmium.

Cadmium has also been shown to activate cell death by necrosis, defined as unexpected or accidental cell death, which can be induced by toxins, radiation, trauma, lack of oxygen and

many other stressors. This mechanism of cell death is more common when cells are exposed to high concentrations of cadmium at one time rather than lower exposures that accumulate over an extended period of time (Lopez et al., 2003). Necrosis also has been shown to be primarily related to cadmium toxicity within the brain and neural tissue (Lopez et al., 2003) as well as in osteoblasts of bone formation (Brama et al., 2012). In this mechanism, mitogen-activated protein kinase (MAPK) pathways are thought to be targeted in both the brain and bone development (Brama et al., 2012). It has not been determined if necrosis is associated with kidney degeneration.

Since ROS production is central to cadmium-induced toxicity as well as a number of other causes of cell death, numerous studies have been initiated to identify methods to protect against its deleterious effects. In 2014, Poljšak and Fink presented two ways that could possibly protect the human body from increases in ROS. The first and most obvious method is physiochemical protection, which simply means to reduce the rate of exposure to the toxic agent. Avoiding the use of lithium batteries or cigarette smoke are examples of how this protective method could be implemented. Obviously, individuals exposed to cadmium in the environment or those subject to industrial byproducts do not have this option. In these cases, Poljšak and Fink proposed that the best mechanism of defense is physiological protection. In this method, the best defense is to increase the presence of antioxidants in the body to better protect against a toxin. There is growing scientific evidence that antioxidants are involved in the prevention of, or the decrease in, cellular damage caused by specific environmental pollutants and carcinogens. Antioxidant intake can easily be increased by consuming specific compounds containing antioxidants, found in various fruits and vegetables.

There are a wide variety of antioxidants found in nature and have been shown to block the harmful effects of ROS by multiple mechanisms. In aerobic organisms, endogenous antioxidant systems exist and can be categorized into enzymatic and nonenzymatic antioxidants. Examples of nonenzymatic antioxidants include vitamins, flavonoids, glutathione, and carotenoids. Flavonoids have specifically been shown to reduce free radical formation as well as scavenge free radicals (Pietta, 2000). Glutathione, on the other hand, detoxifies hydrogen peroxide and lipid peroxides by donating its electrons to hydrogen peroxide, reducing it to water and oxygen particles. Endogenous antioxidants can only regulate a limited amount of ROS, and in pathological situations, these antioxidant systems can be overwhelmed by such vast accumulations of ROS over short periods of time. Therefore, identifying exogenous sources of antioxidants to keep ROS and antioxidant levels in a state of equilibrium during times of oxidative is an area of intense research.

Recently, many folk medicines have been proposed to protect against different diseases that lead to cellular death including diabetes, hypertension, liver malfunction, and the treatment of arsenic poisoning (Dua et al., 2015). Almost all of these folk medicines contain some type of nonenzymatic antioxidant. Most recently, several studies have identified antioxidants that have been shown to have protective effects against cadmium toxicity. Han et al. showed that treating *Iris hexagona,* a rhizomatous perennial, with exogenous salicylic acid protected against cadmium toxicity (2015). In this study, ornamental hydrophyte *Iris hexagona* was treated with cadmium concentrations ranging from 100 μM-500 μM for seven days, and the viability of the plant was analyzed through measurements of dry weight, chlorophyll content, photosynthetic parameters, and thiobarbituric acid reactive substance content (Han et al., 2015). Cadmium treatment for seven days decreased plant viability across all concentrations. However, 1 μM salicylic acid

administered to each plant prior to cadmium treatment was shown to increase plant viability through all concentrations. This protective effect is thought to be due to an increase in plant antioxidant levels before the toxin was introduced (Han et al., 2015).

A second study observed the protective effects of taxifolin, a bioflavonoid found in conifers, against cadmium toxicity in zebrafish (Manigandan et al., 2015). In this study, it was found that embryos exposed to 100 μM cadmium not only had reduced survival, but toxicity also caused delayed hatching and phenotypic abnormalities after fertilization (Maniganden et al., 2015). Cadmium treatment was also shown to increase embryo cardiac function. Embryos treated with concentrations of taxifolin ranging from 0.1 μM-10 μM were more likely to survive and had fewer abnormalities (Maniganden et al., 2015). The study concluded that taxifolin may have the ability to scavenge radicals against damaging oxidative stress, suppressing cadmium's toxic effects (Maniganden et al., 2015).

In 2012, Aktoz et al. studied the protective effects of the flavonoid, quercetin against cadmium toxicity in rat kidney cells. Quercetin is an antioxidant flavonoid found in broccoli, leafy greens, tomatoes, and berries and has been promoted to have anti-cancer effects and improve cellular function (Aktoz et al., 2012). Rats were either treated with 1 mg/kg cadmium chloride for thirty days or administered this same treatment along with 15 mg/kg of quercetin once a day for thirty days, with treatment starting two days prior to cadmium chloride treatment. Kidneys of each rat were then extracted and examined histologically. Rats treated solely with cadmium showed mesangial expansion and thickening capsular, glomerular and tubular basement membranes (Aktoz et al., 2012). In contrast, rats treated with quercetin exhibited only a few swollen glomeruli, and authors saw marked protection of all renal structures when compared to cadmium treated rats. The overall findings of this study suggest that quercetin may

attenuate cadmium-induced renal toxicity, which seems to be operating through the antioxidant effects of quercetin (Aktoz et al., 2012).

Most recently, quercetin has been shown to specifically decrease levels of autophagy in mouse kidney cells by reducing levels of reactive oxygen species (Yuan et al., 2016). Cadmium injected at 0.40 mg/kg/day for three days was shown to have the ability to decrease levels of cellular antioxidants as well as increase levels of reactive oxygen species, which ultimately resulted in induction of autophagy in mouse kidneys. However, when treated with varying concentrations of quercetin (0, 5, 15, 25, 50, 75, and 100 mg/kg/day) for three days, levels of cadmium-induced reactive oxygen species decreased while antioxidant capacity within the kidney was increased at all levels of treatment. These findings allude to the idea that quercetin may be a specific agent to begin examining the production of a treatment for cadmium injury in the kidney (Yuan et al., 2016).

The previous studies support that quercetin and other flavonoids may protect against cadmium toxicity yet the mechanism by which ROS species are regulated is unknown. In a study that examined the effects of two common edible herbs, *Ipomoea aquatica* and *Enhydra fluctuans*, rat liver hepatocytes were treated with cadmium. It was observed that p47phox, one of the six subunits that comprise the multiprotein complex NADPH oxidase, increased with cadmium treatment and was activated by phosphorylation (Dua et al., 2015). Since NADPH oxidase functions in catalyzing the transfer of electrons from NADPH to molecular oxygen, to produce ROS (Sedeek et al., 2013), it is possible that cadmium may, in part, induce ROS production through activation of this enzyme. When rats were fed diets containing *Ipomoea aquatica* and *Enhydra fluctuans,* there was less liver damage as compared to the cadmium treated controls. Further, the investigators showed that these herbs resulted in decreased

amounts of p47phox, and that there was decreased phosphorylation of any p47phox that was expressed in the cells (Dua et. al., 2015). HPLC chromatography of *Ipomoea aquatica* and *Enhydra fluctuans* identified a number of molecules that may be responsible for these protective effects and include the flavonoids quercetin, apigenin, myricetin, ascorbic acid, phenolics, saponins, and various carbohydrates. While the combination of these molecules has been shown to be protective against cadmium toxicity, it is unknown which, if any, of these compounds alone may provide protective affect against cadmium-induced cell death.

While quercetin has been shown to induce protective effects against cadmium toxicity mainly in rats, it has also been observed that various plant extracts containing quercetin protect against cadmium toxicity in varying human cell lines (Sandbichler and Höckner, 2016). Since kidneys are the primary target where cadmium accumulates in the body, this study was designed to determine if quercetin could provide a protective effect against cadmium toxicity using the human embryonic kidney cell line, HEK-293. If observed, this study may spark interest in the use of antioxidants as a useful tool in protecting the human body from the deleterious effects of cadmium and could lead to the development of new therapies to treat against cadmium toxicity.

METHODS

Determining Optimal Concentration of Cadmium-Induced Cell Death using Cell Proliferation Assays

The minimum concentration at which cadmium induces cell death was first established before determining if quercetin has a protective effect against cadmium's toxic effects. HEK-293 cells (ATCC, Manassas, VA) were grown at 37 °C in 5% CO_2 for the entirety of this study. Growth was maintained following manufacturer's directions using Dulbecco's Modified Eagle's Medium 4.5g/L glucose and L-glutamine (DMEM) phenol red (Lonza, Allendale, NJ) supplemented with 10% Fetal Bovine Serum (FBS) (ATCC). In the initial experiments, HEK-293 cells were plated at 5000 cells/well in a 96-well plate for one, two, and four days in 100 μL of DMEM. Cells were then treated with varying concentrations of cadmium chloride ranging from 20 nM-20 mM for one, two, and four days. Since cadmium concentrations were diluted in 10 mM HEPES giving a final concentration of 0.5 mM, the same concentration of HEPES was added to untreated cells. MTT assays using Promega CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Madison, Wisconsin) were then conducted following manufacturer's directions. Absorbance readings at 490 nm were performed using a BioRad 680 plate reader (Hercules, CA). For most studies, incubation at 37 °C for 1.25 hours produced results that were within a linear range of absorption. Analysis of absorbance revealed the ratio of cellular proliferation associated with each concentration of cadmium chloride compared to control, untreated HEK-293 cells.

After five separate trials the experimental design was altered because the HEK-293 cells were growing at a faster rate than the designated media could sustain for the length of these

experiments. Cell proliferation assays were repeated using 1000 cells/well in a 200 μL volume of DMEM supplemented with FBS while keeping overall concentrations of cadmium the same. Since cultures treated for four days must have media and treatment replenished after 48 hours, it became noticeable that the HEK-293 cell line was prone to lifting off of culture plates easily, causing a large percentage of cells to be lost before MTT assay analysis took place. Therefore, cells were only treated for one and two days in the experiments without replacement of media or treatment.

Determining the Protective Effect of Quercetin on Cadmium-Induced Cell Death

Once the optimal concentration of cadmium needed to induce significant cellular death was determined, varying concentrations of quercetin were used to treat cells simultaneously with 30 μM cadmium to see if quercetin had a protective effect against cadmium-induced cellular death. MTT assays were performed as described above. Cells were treated with 10 μM, 100 μM and 200 μM concentrations of quercetin alone or with 30 μM cadmium chloride for 24 hours. Quercetin was dissolved in DMSO, so all treatments contained a final concentration of 1% DMSO as a control.

In a third set of experiments, HEK-293 cells were treated with quercetin 24 hours prior to cadmium exposure, providing time for quercetin to be absorbed by the cells before exposing them to cadmium. In this experiment, HEK-293 cells were treated with 10 μM, 100 μM or 200 μM quercetin for 24 hours before adding 30 μM cadmium chloride to these cells for an additional 24 hours. MTT assays were again used to measure cellular proliferation.

Immunoblot Analysis

HEK-293 cells were plated at 400,000 cells in 6 mL of DMEM supplemented with FBS in 60 mm^3 plates and allowed to adhere for 24 hours. Cells were then treated with 10 μM, 100 μM or 200 μM quercetin for 24 hours and then treated with 30 μM cadmium chloride for 12 hours. The shorter time was to ensure that there would be enough protein present in those cells treated with cadmium chloride for analysis.

Following 12 hours of cadmium chloride treatment, cell extracts were collected and lysed in a cell lysis buffer that contained RIPA, containing 1/1000 phosphate inhibitor III, 1/1000 phosphate inhibitor II, 1/1000 protease inhibitor, and 1mM PMSF. RIPA, phosphate inhibitors, protease inhibitor, and PMSF were all obtained from Sigma Aldrich. To prepare, cells were washed twice with 1-2 mL of ice cold PBS. Plates were then placed on ice and 60 μL of the prepared lysis buffer was added to each plate. Using a BD Falcon cell scraper (VWR, Randor, PA), cells were quickly scraped from the plate, and the cell extract was then collected and placed on ice. All treatments were then sonicated at the lowest setting twice, one second for each sonication. Samples were then spun at 12000 rpm for 10 minutes at 4 °C. The supernatant, containing cellular protein extracts, were transferred to new microcentrifuge tubes and stored at -80°C.

To determine the protein concentration of the cell lysates, Bradford Assays were used following the Bio-Rad protein assay microassay procedure. Samples were measured at 595 nm and normalized with BSA standards. From measured protein lysates, concentration of protein was determined in each sample.

Proteins samples (18.75 µg) were then prepared in Laemmli Buffer (Bio-Rad) and heated for 5 minutes at 95 °C. Samples were then loaded onto a 4-20% polyacrylamide gel with 1X Bio-Rad Running Buffer and run at 200 V for approximately one hour until Laemmli Buffer reached the bottom of the gel. The gel was then removed and proteins were transferred to a Polyvinylidene difluoride (PVDF) membrane at 30 V overnight at 4 °C in transfer buffer (Bio-Rad). The PVDF membrane was then removed from the transfer apparatus, blocked with 5% milk in TTBS (0.1% Tween 20 in Tris-Buffered Saline) for one hour at room temperature. The membrane was then washed once for fifteen minutes and followed by three subsequent five minute washes with TTBS. The membrane was then probed with a 1/500 dilution of polyclonal antibody raised to p47phox (Cell Signaling, Danver, MA) in TTBS containing 5% BSA for three days at 4°C. Washes were repeated as previously described and the membrane was probed with 1/500 dilution of goat anti-rabbit conjugated to horseradish peroxidase as the secondary antibody (Thermo Scientific Waltham, MA) for one hour at room temperature. The membrane was then washed as previously described. Following TTBS washes, Pierce ECL Western Blotting Substrate® (Thermo Scientific) was used for imaging purposes. Fifteen minute exposure of Autoradiograph Film-Blue (World Wide Medical Products Inc., Bristol, PA) to the ECL treated membrane gave optimal results.

This PVDF membrane was then stripped of the primary and secondary antibodies using Restore Western Blot Stripping Buffer™ (Thermo Scientific) and re-blocked with 5% milk in TTBS for one hour at room temperature. The PVDF membrane was then reprobed with 1/5000 dilution of antibody raised to β-actin (Sigma Aldrich, St. Louis, MO) for one hour at room temperature, followed by 1/500 dilution of goat anti-mouse conjugated to horseradish peroxidase as the secondary antibody.

Densitometry

PVDF membrane was imaged using the same method as previously described. Immunoblot analysis was then analyzed by densitometry using Unscan-it software (Silk, Orem, UT) and reported as relative amounts of p47phox to β-actin.

RESULTS

To determine the protective effects of quercetin against cadmium induced toxicity, it was first important to establish the optimal concentration of cadmium that would induce cell death in the HEK-293 cell line used in this study. The aim was to identify a concentration of cadmium chloride that induces 20-30% reduction in growth rather than maximum cell death so that cells would still be viable for further studies. To this end, HEK-293 cells were treated with varying concentrations of cadmium chloride ranging from 10 μM to 50 μM, and proliferative growth was measured using an MTT assay after 24 and 48 hour treatments, (Fig 1 and 2). These experiments were repeated three times each.

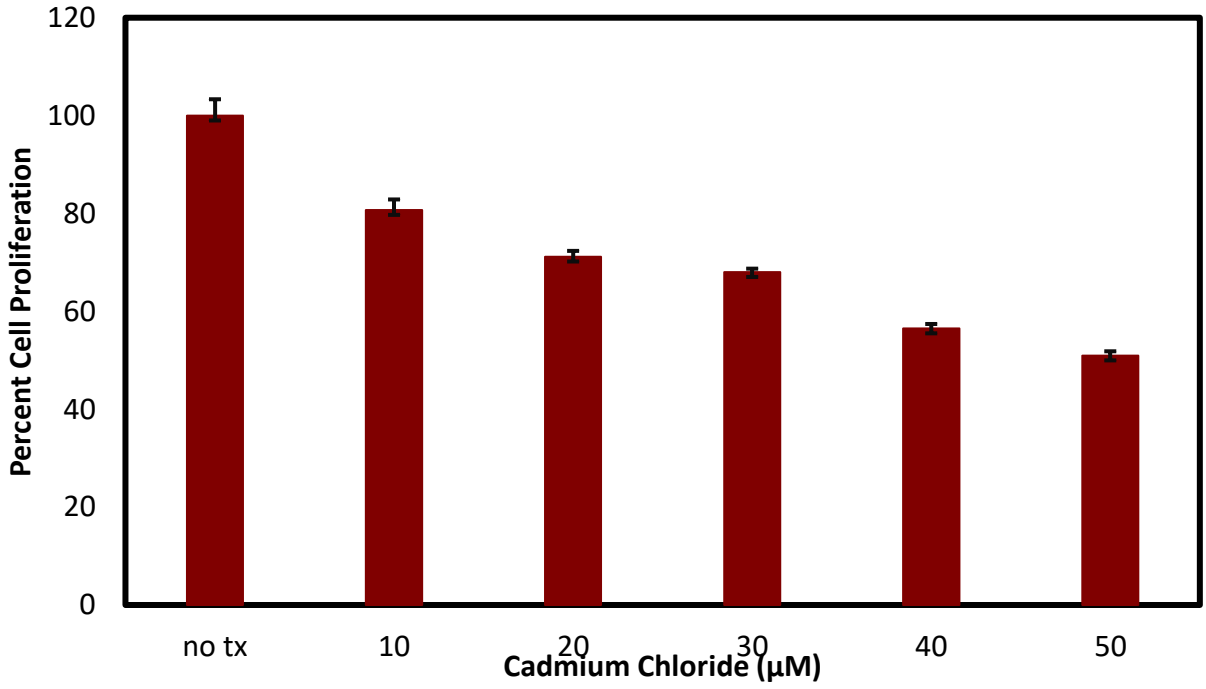


Figure 1: Proliferation of HEK-293 cells is inhibited by cadmium chloride in a concentration dependent manner after 24 hours of treatment. Cells were treated with varying micromolar concentrations of cadmium chloride for 24 hr and proliferation was measured. This experiment was repeated three times.

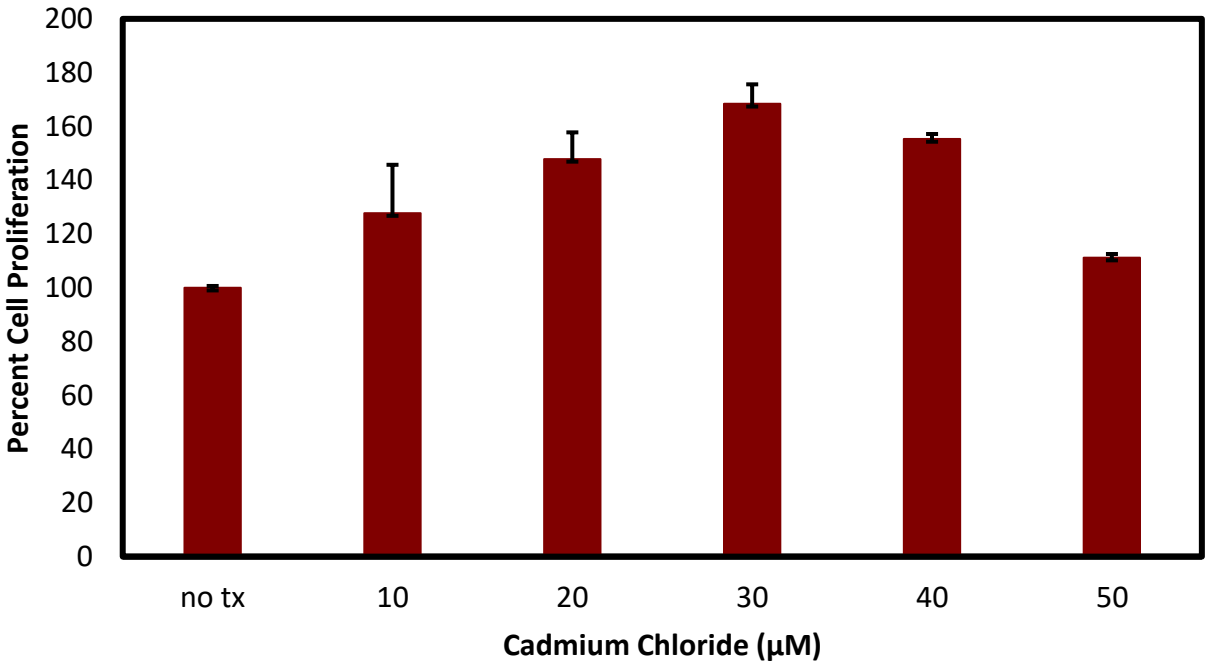


Figure 2: Treatment of HEK-293 cells with varying concentrations of cadmium chloride for 48 hours resulted in unexpected results. Cells were treated with varying micromolar concentrations of cadmium chloride for 48 hr. This experiment was repeated three times.

When cells were treated for 24 hours, it was observed that there was a decrease in cell growth with increasing concentrations of cadmium chloride suggesting a strong correlation between cadmium concentrations and cell death. Further, these results indicated that 30 µM cadmium chloride resulted in a decrease in cell growth by 31.98% (Fig 1). Higher concentrations of cadmium chloride (40 µM and 50 µM) decreased cell growth by 43.49% and 49.02%, respectively, after 24 hour treatment (Fig 1). These results confirmed that cellular proliferation was inhibited by cadmium chloride in a concentration dependent manner. In contrast, treating cells for 48 hours gave surprisingly inconsistent results. As shown in Figure 2, these results suggest that cadmium chloride increased cellular growth with increasing concentrations. Since these results are unexpected, further observations during these

experiments were performed and indicated that there were several factors that could be responsible for this unexpected result.

Viewing the cells under a microscope clearly indicated that the cells were dead suggesting that the proliferation assay was incorrect, because cells treated with the highest concentrations of cadmium chloride were seen to be lifting off of the culture plate after treatment, rather than adhering and proliferating. Secondly, a color change in the DMEM in control cells and cells treated with lower levels of cadmium chloride from red to yellow indicated that cells were using up their allotted DMEM supply before MTT assay analysis. After reviewing the procedure, it was clear that the untreated cells grew so fast that they also died in the 48 hour period due to lack of essential nutrients. Therefore, comparing the cadmium treated cells to the dead, untreated cells was a useless measure after 48 hours. Because of these observations, the remaining experiments in this study were limited to 24 hour cadmium treatments. It was also determined that all following experiments would be performed using 30 μM cadmium chloride to inhibit cellular growth. This concentration is optimal because it is clear that cadmium is having a toxic effect on HEK-293 cells, yet the cells are still able to at least partially respire after 24 hours of treatment. To determine the protective effect of quercetin, it was important that these cells are not past the point of protection, and 30 μM cadmium chloride exhibits each of these desired characteristics.

Once the optimal concentration of cadmium needed to induce toxicity in HEK-293 cells was determined, the next experiment was designed to determine if quercetin had any protective effects. The final concentrations of quercetin used in this study were 10 μM, 100 μM, and 200 μM dissolved in DMSO. These concentrations were chosen because the concentration of quercetin normally found in natural compounds such as leafy greens and bright flower petals is

between 10µM and 25 µM (Koh et al., 2009). Much higher concentrations of quercetin were also used to determine if vast amounts of quercetin must be present to exhibit a protective effect, as well as compare the effects to concentrations present in natural compounds. When HEK-293 cells were treated with these concentrations of quercetin simultaneously with cadmium chloride, quercetin had no effect on cellular growth compared to cadmium chloride treatment (Fig 3). While cadmium chloride treated cells exhibited a 34.61% decrease in proliferation alone, cells treated with quercetin and cadmium chloride simultaneously averaged a similar 39.67% decrease in cellular proliferation.

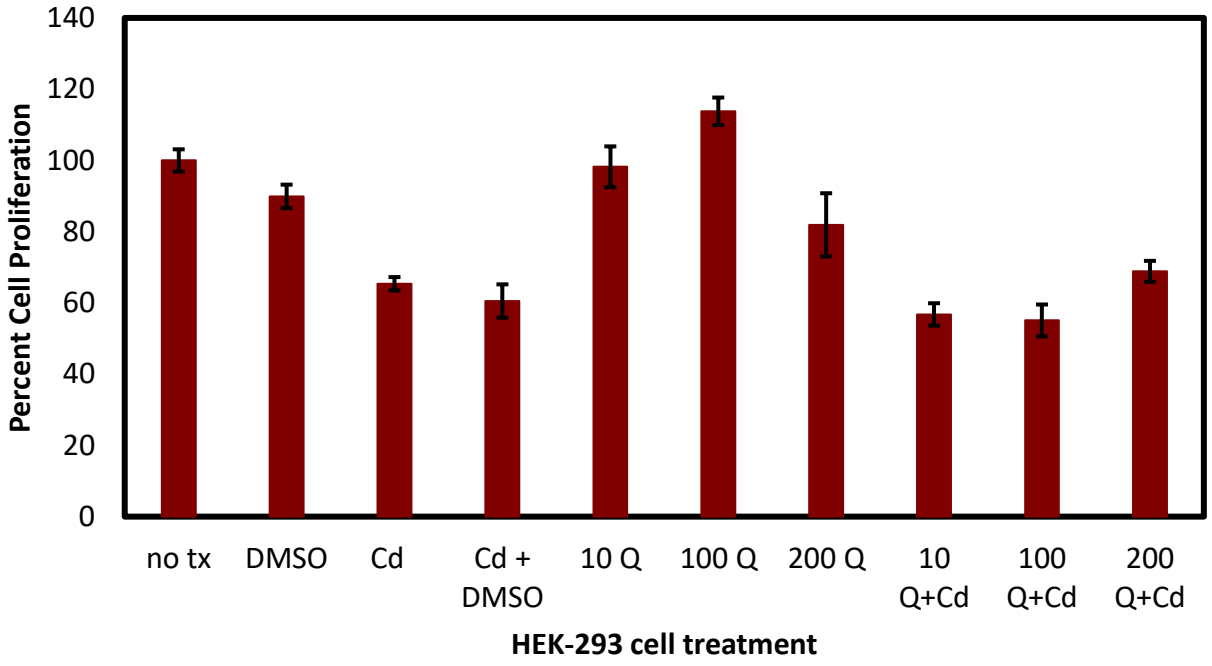


Figure 3: Quercetin does not protect cells from cadmium toxicity when treated with cadmium chloride simultaneously. Cells were treated with varying micromolar concentrations of quercetin and 30 µM cadmium chloride for 24 hr. This experiment was repeated twice.

It is possible that treating simultaneously with quercetin and cadmium does not give quercetin time to inhibit the toxic effects of cadmium. To determine if having quercetin present in the cell before treating with cadmium, this experiment was repeated with the same concentrations of quercetin but cells were treated with quercetin 24 hours prior to treatment with cadmium chloride. In this experiment, it was observed that while cadmium chloride treated cells decreased in cellular proliferation by 34.26% (Fig 4), cells pretreated with quercetin concentrations averaged a 15.87% decrease in cellular proliferation (Fig 4). These results suggest that when HEK-293 cells are primed with quercetin before cadmium toxicity is induced, less cell injury is incurred.

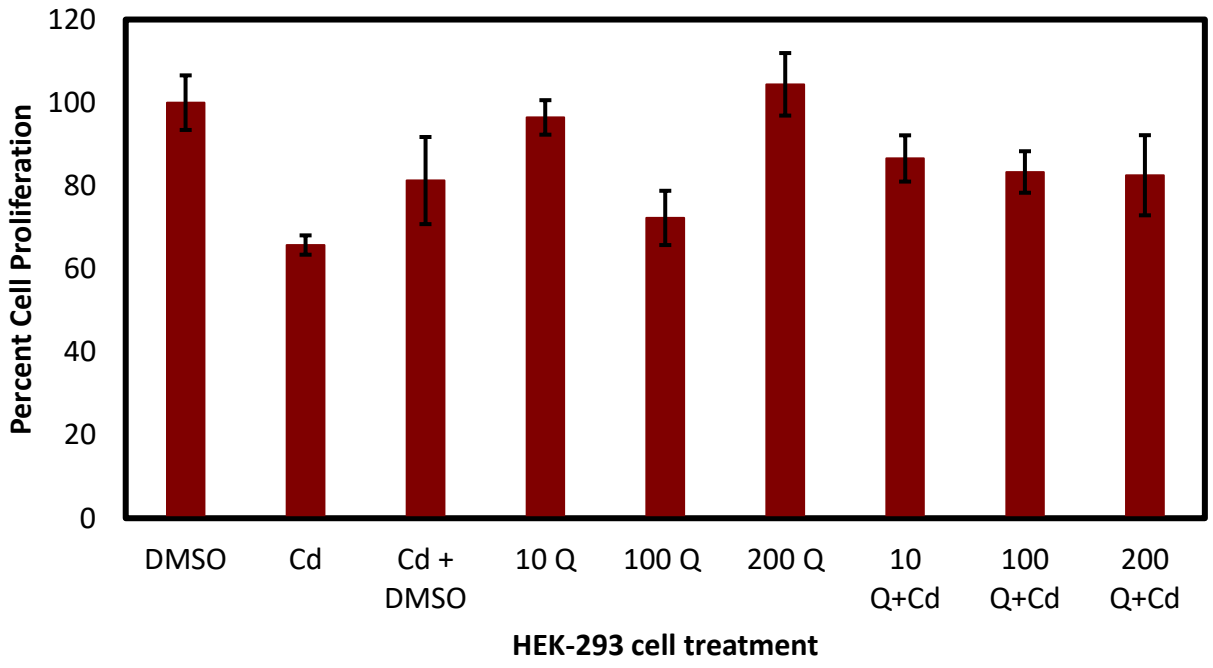
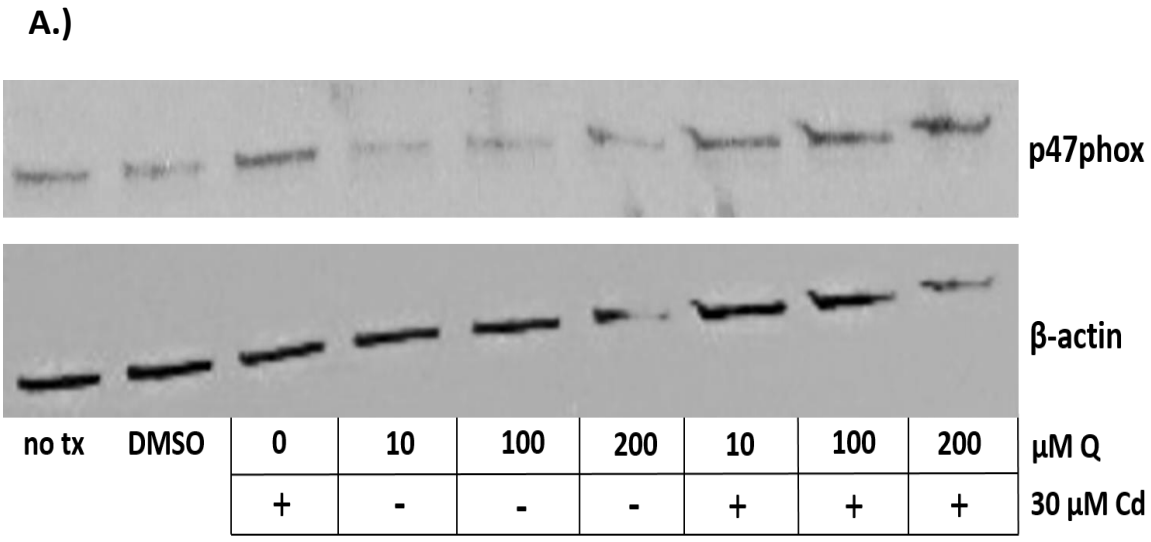


Figure 4: Pretreatment with quercetin for 24 hours partially protects cells from cadmium toxicity. Cells were treated with varying micromolar concentrations of quercetin for 24 hr prior to 30 µM cadmium chloride. This experiment was repeated twice.

Previous studies have shown that p47phox protein, a subunit of NADPH oxidase, increases in cells treated with cadmium. This increase could suggest that cadmium treatment induces an increase in ROS production, as NADPH oxidase ultimately produces superoxides that are converted to ROS. To determine if quercetin may have the ability to inhibit this rise in p47phox, immunoblot analysis was performed. The presence of p47phox was compared in cells treated with cadmium versus those pretreated with these same varying concentrations of quercetin previously studied for 24 hours followed by a 12 hour treatment with 30 μM cadmium chloride. Treatment for 12 hours with cadmium chloride was administered rather than 24 hour treatment to ensure that concentrations of protein would be high enough in all samples to pursue analysis by immunoblot.

Immunoblot analysis of p47phox showed that protein concentrations were increased in cells treated with 30 μM cadmium chloride alone, suggesting that cadmium does increase levels of NADPH oxidase (Fig 5A and 5B). When HEK-293 cells were pretreated with varying concentrations of quercetin followed by cadmium chloride, there was little difference in the cellular levels of p47phox in those treated with 10 μM and 100 μM quercetin suggesting that quercetin does not inhibit cadmium-induced increase in p47phox. Surprisingly, relative ratios of p47phox/β-actin were greatly increased with 200 μM quercetin alone and highly increased in those cells treated with both 200 μM quercetin and cadmium chloride. Since this experiment was performed only once, it is possible that these results are inaccurate. Looking at the levels of the loading control, β-actin, the concentrations are lower in these two treatments compared to all other treatments (Fig 5A). This could be due to an artifact associated with the experimental procedure but this cannot be confirmed until the experiment is repeated. If this result is observed

consistently, it may be that high concentrations of quercetin (200 μM) may have detrimental effects on the cell, altering β-actin levels.



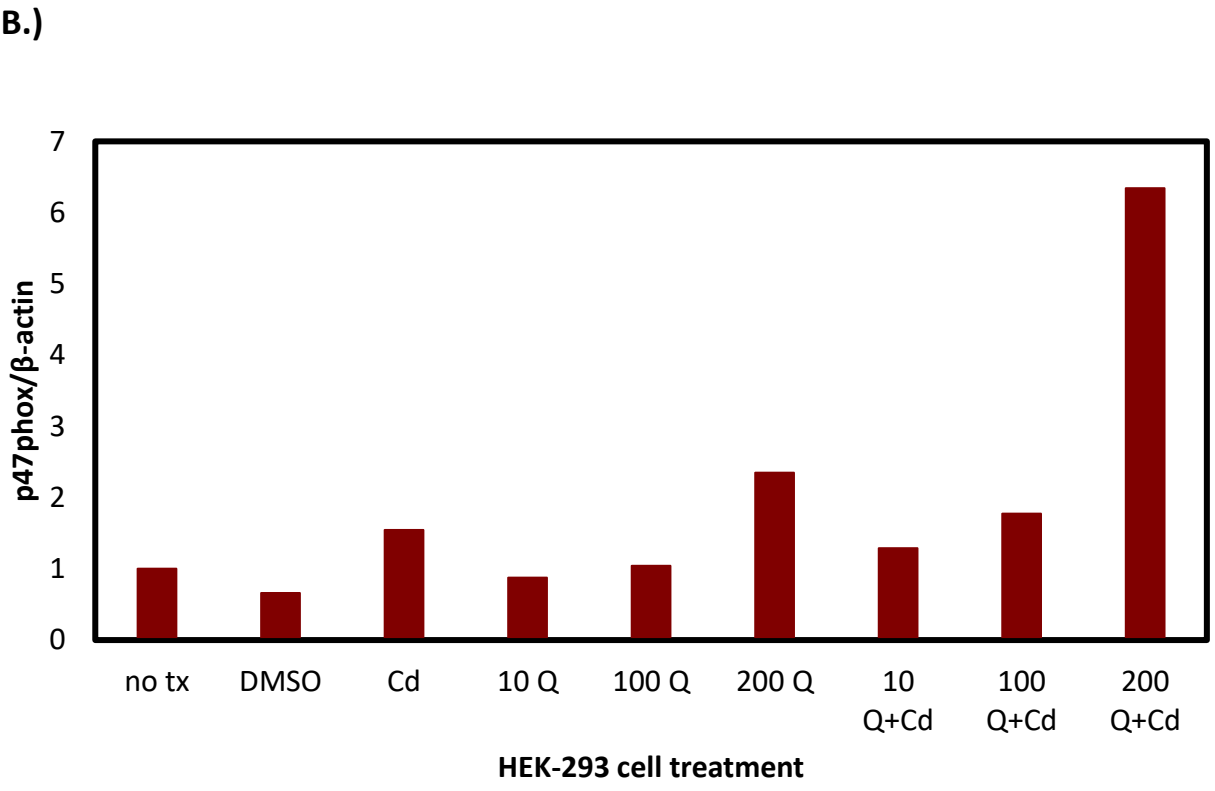


Figure 5: Quercetin does not substantially lower the amount of p47phox in HEK-293 cells. A) Immunoblot analysis of p47phox and β-actin B) Quantification of Immunoblot reported as relative p47phox/β-actin. Cells were treated with micromolar concentrations of quercetin for 24 hr followed by 12 hr treatment with cadmium chloride. This experiment was done once.

DISCUSSION

The first goal of this study was to determine concentrations of cadmium chloride that induces cell death. From this, a concentration of cadmium chloride was found that would decrease cellular proliferation of HEK-293 cells, but also had the potential of being rescued by quercetin. After treating HEK-293 cells for 24 hours with varying concentrations of cadmium chloride, it was determined that cadmium treatment effects cell proliferation in a concentration dependent manner. Through this study, it was concluded that 30 μM cadmium chloride was the optimal treatment for remaining experiments, showing a 31.98% decrease in cellular proliferation after 24 hours.

Once determined, the possible protective effects of quercetin were analyzed with cadmium chloride treatment. It was determined that simultaneous treatment with 30 μM cadmium chloride and varying concentrations of quercetin does not protect cells from cadmium toxicity. Yet, pretreatment with 10 μM, 100 μM, and 200 μM quercetin before cadmium chloride treatment was shown to partially protect cells from cadmium-induced toxicity. With this new information, the study was taken further to analyze a possible mechanism in which cadmium may be triggering cell death and determine if quercetin may protect against it.

The concentration of the p47phox subunit of cadmium and/or quercetin treated cells was analyzed with immunoblot analysis to observe relative levels of p47phox/β-actin. It was first found that p47phox protein expression increases with cadmium treatment when compared to untreated cells. Therefore, cadmium chloride treatment could be increasing NADPH oxidase levels that could then allow for increased ROS production leading to cellular death. However, pretreatment with quercetin does not reduce the levels of p47phox induced by cadmium. While

cadmium may be operating through p47phox to induce cellular death, this is most likely not the mechanism that quercetin is working through as a means of protection.

Immunoblot analysis of p47phox was only performed once, so conclusions cannot be made until this experiment is repeated several times. Another unusual observation was the increase in p47phox levels in cells treated only with 200 µM quercetin as well as those treated with 200 µM quercetin and 30 µM cadmium chloride. While no conclusions may be drawn, there are several possible explanations for this unusual observation.

It has been found that high concentrations of quercetin may have a negative impact on cellular proliferation. Research shows that high concentrations of quercetin, such as 200 µM, may have a role in DNA damage as well as apoptosis (Watjen et al., 2005). One specific research study demonstrated that H4IIE rat hepatoma cells treated with varying concentrations of quercetin could protect against peroxide induced cell damage as well as induce cellular damage, depending on concentration (Watjen et al., 2005). Concentrations of quercetin at 10-25 µM showed minimal cellular damage with peroxide-induced cell death whereas concentrations of quercetin at higher concentrations ranging from 50-250 µM alone was shown to induce cytotoxicity.

While these findings seem to appear similar to the results of the experiments with HEK-293 cells reported here, literature searches do not provide much more insight on why higher concentrations of quercetin may be cytotoxic to human cell lines. This is one area of study that should be explored before flavonoids such as quercetin are hailed as miracle antioxidants.

There are multiple directions that this study could follow to identify a mechanism that quercetin may use to protect HEK-293 cells from cadmium toxicity. Although quercetin was not

seen to decrease levels of p47phox in cadmium treated cells, this does not specifically indicate that ROS production is increasing. Studies have shown that for p47phox to be active it must be phosphorylated. A second experiment using immunoblot analysis probing for phosphorylated p47phox would be one way to determine if p47phox is active in cadmium as well as quercetin treated cells. Results will support the hypothesis that p47phox is a part of the pathway that quercetin is using to protect against cadmium toxicity.

Secondly, ROS production in HEK-293 cells could be determined through a ROS specific assay. ROS levels within a cell can be quantified relative to untreated cells, similar to cell growth quantification shown by MTT assays. If ROS levels are quantified in HEK-293 cells treated with cadmium and quercetin, it may demonstrate if either substance is having an effect on total ROS levels that could be contributing to or inhibiting cell death.

If ROS assays show quercetin does have a protective affect against increased ROS production, but p47phox activation does not decrease with quercetin treatment, it may be that a different signaling mechanism is involved. c-Jun-N-Terminal kinases (JNKs) are another possible target of cadmium as well as quercetin. Three isoforms of JNKs have been shown to be activated in response to varying cellular stresses including oxidative stress, DNA-damaging agents, protein synthesis inhibitors as well as other stressors (Cargnello and Roux, 2011). Cadmium exposure to the kidney may cause a number of cellular stressors, which could induce phosphorylation of JNK, leading to cellular apoptosis. Whichever path this study may follow, the ultimate goal is to find a protective agent against cadmium toxicity as well as the mechanism that it works through to help find a treatment for those subject to chronic kidney disease caused by cadmium.

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